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Award Number: W81XWH-04-1-0047

TITLE: Role of Tumor Stroma in Prostate Carcinogenesis

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REPORT DATE: March 2005

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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20050712 077

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY
(Leave blank)**2. REPORT DATE**
March 2005**3. REPORT TYPE AND DATES COVERED**
Annual Summary (15 Feb 04 - 14 Feb 05)**4. TITLE AND SUBTITLE**

Role of Tumor Stroma in Prostate Carcinogenesis

5. FUNDING NUMBERS

W81XWH-04-1-0047

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REPORT NUMBER****9. SPONSORING / MONITORING
AGENCY NAME(S) AND ADDRESS(ES)**U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012**10. SPONSORING / MONITORING
AGENCY REPORT NUMBER****11. SUPPLEMENTARY NOTES****12a. DISTRIBUTION / AVAILABILITY STATEMENT**

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE**13. ABSTRACT (Maximum 200 Words)**

This project ultimately aims to identify the role of tumor stroma in inducing prostate carcinogenesis. The hypothesis to be tested is that tumor stroma, in the form of carcinoma-associated fibroblasts (CAFs) isolated from human prostate cancer patients, can initiate malignant transformation in human embryonic stem cells.

To date, we have made significant progress towards generating prostatic fibroblast / human embryonic stem cell tissue recombinants. We have taken some time to get ethics approval for the collection of human prostate tissues and subsequent recruitment of patients in collaboration with clinicians. Upon collection of prostate tissues, we have successfully isolated and cultured prostatic fibroblasts. We are currently refining our collection technique to be 100% confident that the fibroblasts we collect are of a malignant phenotype before we claim to have isolated "carcinoma-associated fibroblasts (CAFs)". Meanwhile, we have established the culture of human embryonic stem cells. Importantly, we have gained access to constitutively expressing-GFP human embryonic stem cells for use in tissue recombination experiments. Using these cells, we have generated a number of tissue recombinants that are currently grafted under the kidney capsule of immune-deficient mice. The first round of grafts are due to be harvested in April 2005.

Whatever the outcome of these experiments is, we are sure to contribute significantly to our understanding of the role of tumor stroma in the process of prostate carcinogenesis.

14. SUBJECT TERMSEmbryonic Stem Cells, Prostate Cancer, Mesenchyme,
Tissue Recombination**15. NUMBER OF PAGES**

13

16. PRICE CODE**17. SECURITY CLASSIFICATION
OF REPORT**

Unclassified

**18. SECURITY CLASSIFICATION
OF THIS PAGE**

Unclassified

**19. SECURITY CLASSIFICATION
OF ABSTRACT**

Unclassified

20. LIMITATION OF ABSTRACT

Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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INTRODUCTION

This project ultimately aims to identify the role of tumor stroma in inducing prostate carcinogenesis. To date, the contribution of prostate tumor stroma to the carcinogenic process are equivocal. This project seeks to test the hypothesis that tumor stroma, in the form of carcinoma-associated fibroblasts (CAFs) isolated from human prostate cancer patients, can initiate malignant transformation in human embryonic stem cells. If this hypothesis is proven, then tumor stroma will be implicated in initiating prostate cancer and future work should attempt to dissect the contribution of specific stromal elements to initiation of tumorigenicity. In addition, we will provide evidence that the tumor stroma is a potential target for prostate cancer therapies, we will generate a new model of human prostate cancer that will be available to test prevention strategies, and/or novel therapeutics. Alternatively, if this hypothesis is not correct, then this study will provide evidence that epithelial insult together with changes to the stromal environment lead to malignant transformation. Nonetheless, such information will greatly enhance our understanding of the carcinogenic process. To date, we have made significant progress towards generating prostatic fibroblast / human embryonic stem cell tissue recombinants, as discussed below.

BODY

Task 1: Collection of normal prostatic fibroblasts (NPFs) and carcinoma-associated prostatic fibroblasts (CAFs) and establishment of hES cells [Months 1-6].

- a. Patient recruitment and tissue collection from men with prostate cancer at radical prostatectomy, for collection of carcinoma-associated prostatic fibroblasts (CAFs).*

We have completed the aims listed in Task 1a during the first year of the project. Specifically, this involved patient recruitment and tissue collection of CAFs from men with prostate cancer.

Before this work began, we were required to obtain Human Ethics Approval to access human prostate tissues from patients undergoing radical prostatectomy surgery at Monash Medical Centre in Melbourne, Australia. Primary approval was granted from the Southern Health Human Research Committee C (Approval Number: 03157B) on 6th February 2004 [see appendix 1.1]. This approval covered the procedures to be undertaken at Monash Medical Centre. Secondary approval was granted from the Monash University Standing Committee on Ethics in Research Involving Humans (Approval Number: 2004/145MC) on 22nd April 2004 [see appendix 1.2], which was necessary since some staff involved in the project are employees of Monash University.

Once approval was granted, patient recruitment and tissue collection was done in collaboration with Mr. Mark Frydenberg (*Clinical Director, Centre for Urological Research, Monash Institute of Reproduction & Development; Chairman, Department of Urology, Monash Medical Centre*). To date, we have collected tissue from n=3 patients. This was done using a biopsy technique

where the tumor is palpated in the prostate once it is surgically removed and 10-15 biopsy samples are taken from the 'tumor region'. Upon discussion with several pathologists, it is evident that this technique only yields approximately 40-50% of tissue that is from a malignant region; since the tumor is often small and localized, the chance of collecting tissue from adjacent non-malignant regions is fairly high. Therefore, we are currently establishing a technique where the pathologist is actively involved in the collection of tumor tissue pieces. The pathologist will expose the suspected tumor region and remove a sample of tissue. The pathology of the collected tissue will be confirmed using frozen sections of adjacent tissues. This will provide us with tissue samples that we will confidently know are true samples of malignant prostate tumor from which to isolate carcinoma-associated fibroblasts (CAFs).

At present however, we have proceeded with the isolation, culture and recombination with prostatic fibroblasts collected from human prostate cancer patients using the biopsy technique. For details, see the progress towards **Task 1c**.

b. Patient recruitment and tissue collection from men undergoing non-prostate related urological surgery in which there is no histopathological evidence of carcinoma of the prostate, for collection of normal prostatic fibroblasts (NPFs).

To date, we are still working towards completing Task 1b. Although we have Human Ethics Approval to collect tissue from non-prostate related urological surgical patients, we have not actively recruited patients for this purpose as yet. The main reason for this is that we intend to collect normal prostatic fibroblasts (NPFs) from adjacent non-malignant regions of the prostates used for the collection of CAFs. This will be possible when the pathologist is actively collecting CAFs from malignant tumors using frozen sections for confirmation; he will then collect non-malignant tissue from an adjacent region. This will be a better control since the CAFs and NPFs will be collected from the same patient.

c. Establishment of culture of both normal prostatic fibroblasts and CAFs.

After collection of tissues from the 'tumor region' of surgically removed prostates by multiple biopsy's, we isolated fibroblasts using the technique described in Olumi *et al.*, (1999). Although we are not 100% confident that these cells are of malignant phenotype, we have been able to reproducibly isolate and culture fibroblasts from prostate cancer tissue samples, which will be important when we collect future samples in collaboration with the pathologist. Previous reports indicate that primary cultures of CAF cells are indistinguishable from normal prostatic fibroblasts morphologically, immunocytochemically, and by growth characteristics (Olumi *et al.*, 1999), so it is not possible to identify the phenotype (benign or malignant) of the fibroblasts we have prepared in culture.

Nonetheless, using enzymatic digestion and centrifugation, we have been able to isolate human prostate fibroblasts. These cells are immuno-positive for the fibroblast maker, vimentin (**Figure**

1A) and immuno-negative for smooth muscle α -actin (**Figure 1B**) or epithelial cell markers high molecular weight cytokeratins (**Figure 1C**) or cytokeratins 8/18 (**Figure 1D**).

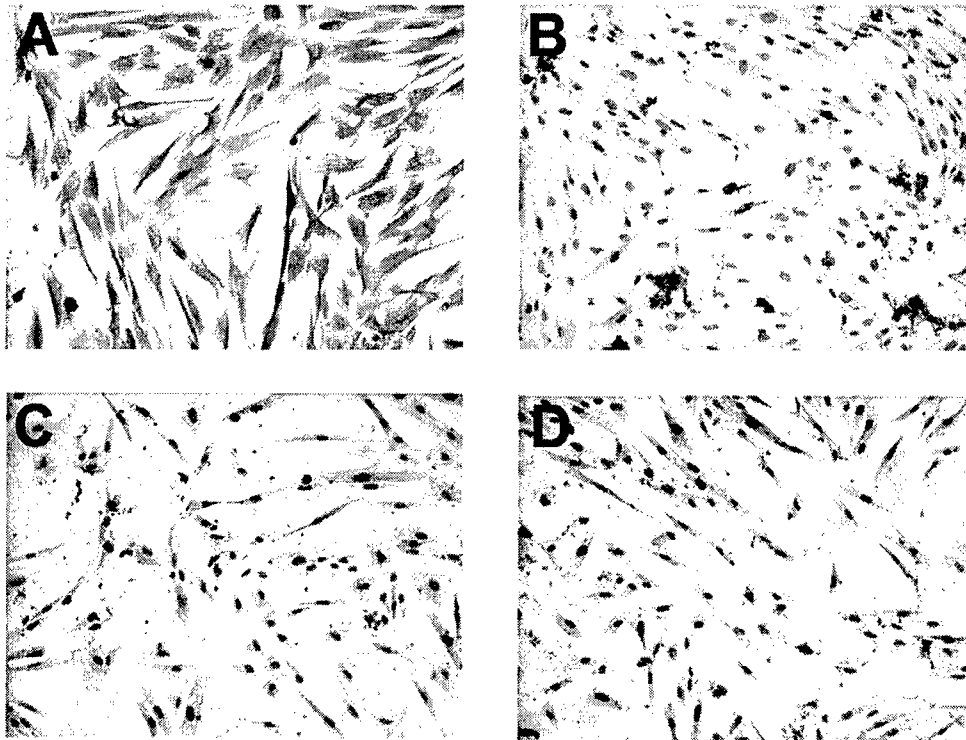


Figure 1 – Prostate fibroblasts isolated from human prostate cancer patients. **A.** Immunolocalisation of vimentin; fibroblast marker. **B.** Immunolocalisation of smooth muscle α -actin; smooth muscle marker **C.** Immunolocalisation of high molecular weight cytokeratins; basal epithelial cell marker. **D.** Immunolocalisation of cytokeratins 8 & 18; luminal secretory epithelial cell marker. *N.B. There is non-specific staining in images B, C and D; this is simply background and is not representative of positive immuno-labelling.*

These cells are well established in culture and have been used in tissue recombination experiments (**Task 2b**).

d. Examination of growth characteristics and properties of CAFs compared to normal prostatic fibroblasts using immunohistochemistry.

There has been no progress towards this aim at the present time since we have not isolated normal prostatic fibroblasts. This will be done in the coming 2-3 months.

e. Establishment and optimization of hES cell cultures on feeder layers to maintain hES cells in undifferentiated state.

We have established the growth of two hES cell lines in our laboratory; hES 2 (genetically female; XX) and hES 4 (genetically male; XY). These cells grow in colonies on a bed of mouse fibroblasts as shown in **Figure 2a**. During propagation, these colonies are cut into 'transfer pieces' as shown in **Figure 2b**. Part of these transfer pieces are used in the tissue recombination studies. Using immuno-fluorescence, we were able to demonstrate that the hES cell colonies express Oct-4 **Figure 2c**; a marker of undifferentiated embryonic stem cells, confirming that continued culture of these cells was maintained in the absence of spontaneous differentiation.

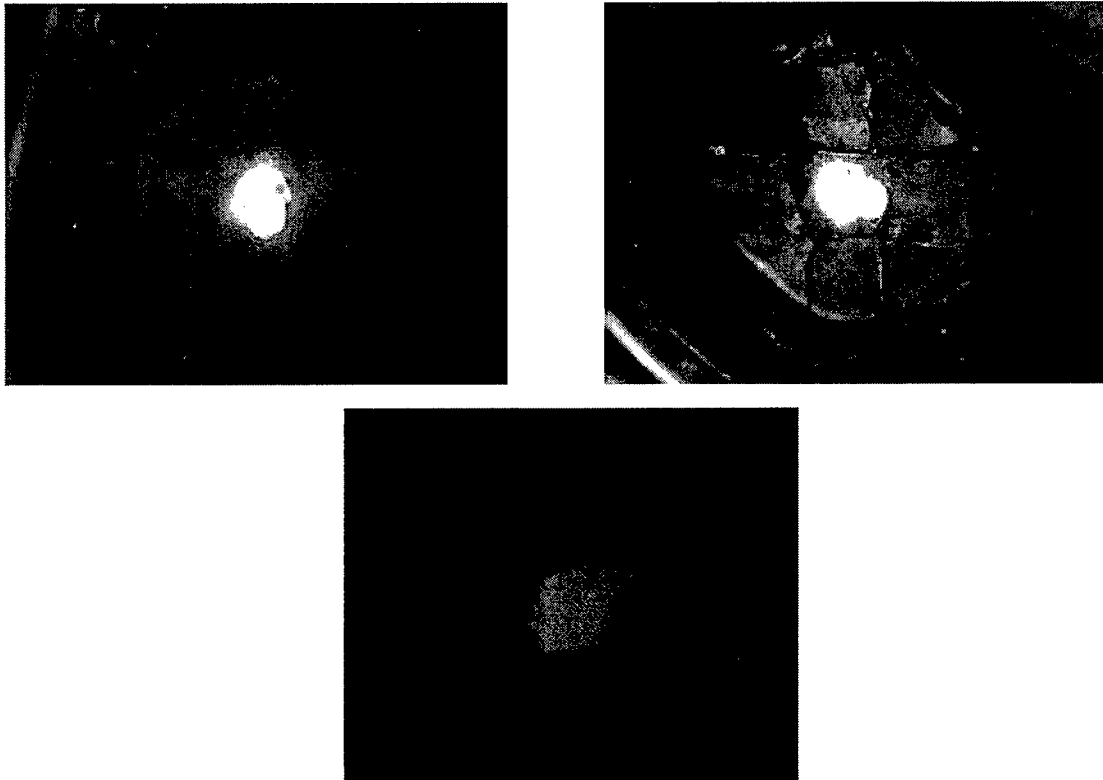


Figure 2 –Human embryonic stem cell colonies. *a. hES 2 colony growing on a mouse embryonic fibroblast layer. b. Transfer pieces of a hES 2 colony in preparation for tissue recombination experiments. c. Immuno-labelling of Oct-4 in undifferentiated human embryonic stem cells.*

Most recently, we have gained access to a new human embryonic stem cell line; ENVY. This is a hES cell line that constitutively expresses green-fluorescent protein (GFP) in both undifferentiated and differentiated states (Costa *et al.*, 2004). This cell line is extremely valuable for this project in order to establish the origin of potential prostate tumor cells in harvested grafts. Since the prostatic fibroblasts and the embryonic stem cells are both of human origin, the GFP label will be able to distinguish cells of hES cell origin, as opposed to potential epithelial cells contamination in fibroblast preparations. These cells have been used in tissue recombination studies discussed in **Task 2b**.

Task 2: Recombination of hES cells with CAF cells [Months 6-18]

- a. Continue growing hES cells and CAF cells in culture.*

This task has been completed successfully and is on-going in the laboratory.

- b. Recombination in vitro using collagen gel technique, followed by subrenal grafting in athymic mice at 3 time points: 4 weeks (1 month), 12 weeks (3 months) and 36 weeks (9 months) to test neoplastic and metastatic capabilities.*

Work towards this task has begun. Early in 2005, we generated the first set of tissue recombinants. The recombinants consisted of human embryonic stem cells, mostly of the ENVY cell line, but also hES 2 and hES 4 cells cultured in our laboratory, in combination with prostatic fibroblasts isolated from human prostate cancer patients. The first grafts are due to be harvested during April 2005.

These experiments will be repeated once CAFs of known phenotype are isolated in the coming weeks. The grafts will be harvested after 4, 12 and 36 weeks to test the neoplastic and metastatic capabilities of hES cells in the presence of CAFs. Experiments with NPFs and hES cells will also be conducted.

- c. Measure wet weights and prepare tissues for analysis.*

There has been no tissue recombinants harvested to date, so we have been unable to conduct any analysis.

Task 3: Morphological analyses of tumorigenesis [Months 18-24]

- a. Immunohistochemistry for prostate specific antigen (PSA), androgen receptor (AR), cytokeratins (CK; including CK8, 18, 5, 14) and smooth muscle actin will show the extent of prostate differentiation and malignant transformation.*

No progress to date.

- b. Tumorigenic markers of cell proliferation (immunolocalisation of proliferating cell nuclear antigen; PCNA) and cell death (by apoptosis) will also be determined and quantitated.*

No progress to date.

KEY RESEARCH ACCOMPLISHMENTS

List of key research accomplishments emanating from this research:

- Gained Human Ethics approval for the collection of human prostate tissues.
- Isolated fibroblasts from human prostate cancer patient samples.
- Cultured and characterized human prostate fibroblasts.
- Established culture of hES 2 and hES 4 cells in the laboratory.
- Made tissue recombinants consisting of prostatic fibroblasts and human embryonic stem cells and grafted under the kidney capsule of immune-deficient mice. Grafts yet to be harvested.

REPORTABLE OUTCOMES

	<i>Reportable outcomes that have resulted form this research:</i>
Manuscripts	Nil.
Abstracts Presentations	Taylor RA, Cunha GR, Trounson AO, Risbridger GP (2005) Stromal microenvironment influences stem cell differentiation in normal and malignant prostate. <i>17th Lorne Cancer Conference</i> , Phillip Island, Australia. (Oral presentation) [See appendix 2]
Patents and licences	Nil.
Degrees obtained	Nil.
Development of cell lines	Nil.
Tissue or serum repositories	Nil.
Informatics such as databases and animal models	Nil.
Funding applied for based on this work supported by this award	Nil.
Employment or research opportunities applied for and/or received based on experience/training supported by this award	Nil.

CONCLUSIONS

In summary, we have made significant progress towards generating prostatic fibroblast / human embryonic stem cell tissue recombinants. We have taken some time to get ethics approval for the collection of human prostate tissues and subsequent recruitment of patients in collaboration with clinicians. Upon collection of prostate tissues, we have successfully isolated and cultured prostatic fibroblasts. We are currently refining our collection technique to be 100% confident that the fibroblasts we collect are of a malignant phenotype before we claim to have isolated "carcinoma-associated fibroblasts (CAFs)".

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Whatever the outcome of these experiments is, we are sure to contribute significantly to our understanding of the role of tumor stroma in the process of prostate carcinogenesis.

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Costa, M., Dottori, M., Stadler, E., Hawes, S.M., Sourris, K., Jamshidi, P., Pera, M.F., Elefanty, A.G., Stanley, E.G. (2004). Envy, A GFP-Expressing Human Embryonic Stem Cell Line. *The Australian Health and Medical Research Congress*, Sydney, New South Wales, Australia.

Olumi, A. F., Grossfeld, G. D., Hayward, S. W., Carroll, P. R., Tlsty, T. D., and Cunha, G. R. (1999). Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. *Cancer Res* **59**, 5002-11.

APPENDIX 1.1: HREC APPROVAL

Southern Health

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Victoria
Australia

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Clayton Victoria 3168
Australia

Human Research Ethn
Secretariat office
tel (03) 9594 3025
fax (03) 9594 6306

HUMAN RESEARCH ETHICS COMMITTEE C CERTIFICATE OF APPROVAL

DATE 06 February 2004
PROJECT NO. 03157B
PROJECT TITLE Role of tumor stroma in prostate carcinogenesis
Patient Information Sheet Version No. 2 dated 10
December 2003
Consent Form Version No. 2 dated 10 December 2003
INVESTIGATOR(S) Dr R Jarred
HREC MEETING DATE 20.11.03
APPROVAL 06.02.2004 – 06.02.2007

The Principal Investigator is required to notify the Executive Officer of the Human Research Ethics Committee of:

1. Any change in protocol and the reason for that change together with an indication of ethical implications (if any)
2. Adverse effects of project on subjects and steps taken to deal with them
3. Any unforeseen events
4. Any expiry of the insurance coverage provided in respect of sponsored trials

At the conclusion of the project or every twelve months if the project continues, the Principal Investigator is required to complete and forward an annual report to the Committee.

Annual report forms will be forwarded to the researcher.

SPECIAL CONDITIONS

SIGNED


.....
Committee Representative

DATE 06 February 2004

Please quote Project No. and Title for all correspondence

Southern Health

ABN 82 142 080 338

Dandenong Hospital
Kingston Centre
Cranbourne Integrated
Care Centre

Monash Medical Centre
Hampton Rehabilitation
Hospital

www.southernhealth.org.au

Community Health
Services across the
South East

APPENDIX 1.2: SCERH APPROVAL

MONASH University



22 April 2004

Dr Renea Jarred
Centre for Urological Research – MIRD
Monash Medical Centre

Prof Gail Risbridger
Institute of Reproduction & Development
Monash Medical Centre

2004/145MC - Role of tumor stroma in prostate carcinogenesis

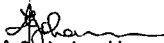
The above research project has been considered by the Standing Committee on Ethics in Research Involving Humans and approval has been given. This approval will be ratified at meeting A3/2004 on 4 May 2004. It is possible that issues may be raised by the Committee at that meeting. If you do not hear anything further you may assume that approval for the project is confirmed.

Terms of approval

1. This project is approved from 22 April 2004 to 6 February 2007 and this approval is only valid whilst you hold a position at Monash University.
2. It is the responsibility of the Chief Investigator to ensure that all information that is pending is forwarded to SCERH. You will then receive a letter from SCERH confirming that we have received a letter from each organisation.
3. It is the responsibility of the Chief Investigator to ensure that all investigators are aware of the terms of approval and to ensure the project is conducted as approved by SCERH.
4. You should notify SCERH immediately of any serious or unexpected adverse effects on participants or unforeseen events affecting the ethical acceptability of the project.
5. **Amendments to the approved project:** Changes to any aspect of the project require the submission of a Request for Amendment form to SCERH and must not begin without written approval from SCERH. Substantial variations may require a new application.
6. **Future correspondence:** Please quote the project number and project title above in any further correspondence.
7. **Annual reports:** Continued approval of this project is dependent on the submission of an Annual Report. Please provide the Committee with an Annual Report determined by the date of your letter of approval.
8. **Final report:** A Final Report should be provided at the conclusion of the project. SCERH should be notified if the project is discontinued before the expected date of completion.
9. **Monitoring:** Projects may be subject to an audit or any other form of monitoring by SCERH at any time.
10. **Retention and storage of data:** The Chief Investigator is responsible for the storage and retention of original data pertaining to a project for a minimum period of five years.

All forms can be accessed at our website www.monash.edu.au/resgrant/human-ethics

We wish you well with your research.


Dr Andrea Lines
Human Ethics Officer (on behalf of SCERH)

Standing Committee on Ethics in Research Involving Humans
Research Grants and Ethics Board
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www.monash.edu.au/resgrant/human-ethics

APPENDIX 2: ABSTRACT PRESENTATION

1. *Oral presentation given by Renea Taylor (nee Jarred) at the 17th Lorne Cancer Conference, Phillip Island, Victoria, Australia (February 2005)*

Stromal Microenvironment Influences Stem Cell Differentiation in Normal and Malignant Prostate

R. A. Taylor¹, G. R. Cunha³, A. O. Trounson², G. P. Risbridger¹

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² *Monash Immunology and Stem Cell Laboratory, Monash University, Clayton, VIC, Australia*

³ *Department of Anatomy, University of California, San Francisco, San Francisco, CA, United States*

The reciprocal interactions between stroma and epithelia of the prostate are critical for the development and normal function of the prostate gland. Prostate cancer is believed to arise from the sequential disruption of these interactions. In order to develop a novel model system to explore the biological mechanisms of prostate development and malignant transformation, we developed a system to differentiate human epithelial cells in vivo. In this study, human embryonic stem cells (hESCs) were used as a renewable reproducible source of epithelial cells. Directed differentiation of hESCs was conducted using tissue recombination. Briefly, hES2 and hES4 cells (ES Cell International, Singapore) were grafted under the kidney capsule of adult male SCID mice for 4, 8 and 12 weeks alone or in combination with neonatal mouse prostate mesenchyme, after which time tissues were collected for pathological and immunohistochemical analysis. When grafted alone hESCs formed teratocarcinomas, whereas when grafted in combination with neonatal prostate mesenchyme, grafts consisted of ductal structures that were histologically indistinguishable from immature human prostate. Androgen receptor was expressed in stromal and epithelial cells, characteristic of human prostate. After 12 weeks, grafts showed signs of maturation as evident by ductal glands lined by a pseudostratified columnar epithelium composed of a full complement of epithelial cells as well as lumen formation and evidence of secretory products. The surrounding stroma was organised in a multilayered concentric ring around the cords indicating the reciprocal nature of the interactions between the stroma and epithelia during differentiation. Therefore, we successfully directed the differentiation of hESCs into prostate epithelium in vivo. This model system is a reproducible reliable means to study stroma-stem cell interactions in normal and malignant tissues. In addition, it provides a novel screening tool to identify therapeutic agents that target the tumor microenvironment.